

Dysregulated primary hemostasis in critically ill COVID-19 patients

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Abstract

Background: Microvascular thrombosis, as well as arterial and venous thrombotic events, have been largely described during severe Coronavirus disease 19 (COVID-19). Therapeutic anticoagulation has been proposed in critical patients, however mechanisms underlying hemostasis dysregulation remain unclear.

Methods: We explored two independent cross-sectional cohorts to identify soluble markers and gene-expression signatures that discriminated COVID-19 severity and outcomes.

Results: We found that elevated soluble (s) P-selectin at admission was associated with disease severity. Elevated sP-selectin was predictive of intubation and death (ROC AUC = 0.67, $p = 0.028$ and AUC = 0.74, $p = 0.0047$, respectively). An optimal cutoff value of 150 NC (normalized concentration) was predictive of intubation with 66% negative predictive value (NPV) and 61% positive predictive value (PPV), and of death with 90% NPV and 55% PPV. Next, an unbiased gene set enrichment analysis revealed that critically ill patients had increased expression of genes related to primary hemostasis. Hierarchical clustering identified *ITG2AB*, *GP1BB*, *PPBP* and *SELPLG* to be upregulated in a grade-dependent manner. ROC curve analysis for the prediction of mechanical ventilation was significant for *SELPLG* and *PPBP* (AUC = 0.8, $p = 0.046$ for both markers). An optimal cutoff value for *PPBP* was predictive of mechanical ventilation with 100% NPV and 45% PPV, and for *SELPLG* was predictive of mechanical ventilation with 100% NPV and 50% PPV.

Conclusion: We provide evidence that platelets contribute to disease severity with the identification of sP-selectin as a biomarker for poor outcome. Transcriptional analysis identified *PPBP* and *SELPLG* RNA count as biomarkers for mechanical ventilation. These findings provide rationale for novel therapeutic approaches with antiplatelet agents.

Background

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent of the coronavirus disease 2019 (COVID-19) pandemic. Severe disease is characterized by an acute respiratory distress syndrome (ARDS), respiratory failure and ultimately death in about 1% of cases (1,2). Most important risk factors for severe disease include age, overweight, diabetes, hypertension and history of cardiovascular disease (3,4). Severe and critical patients were shown to develop arterial and venous thrombotic complications such as pulmonary embolism, stroke and myocardial infarction (5-8). Markers of coagulation activation, in particular increased D-dimer, fibrinogen and von Willebrand factor (vWF) levels were found to be associated with critical illness, whereas only minor changes were noted in prothrombin time and platelet counts (9,10). Additionally, autopsy series described multiple thrombosis in deceased COVID-19 patients (11,12). These findings suggest vascular microthrombotic disease as a primary factor for mortality in critically ill COVID-19 (6,13). Therefore, some authors supported the systematic use of

curative anticoagulation upon admission to intensive care unit (ICU) (14), a strategy that has been reported to decrease mortality in severe COVID-19 (9).

Mechanisms underlying increased thrombotic events are still unclear but accumulating evidence point to a key role for endothelial and platelet activation (15,16). As viral inclusions were described in endothelial cells, it has been hypothesized that endothelial cell injury and activation could drive platelet activation and subsequent coagulopathy (12,17). Therefore, identifying the contribution of platelets to critical illness during SARS-CoV-2 infection is key to identify biomarkers predicting clinical outcome and discuss novel therapeutic strategies.

Platelet P-selectin is a key thromboinflammatory molecule involved in platelet activation and function. It has been demonstrated to play a crucial role in primary hemostasis by regulating platelet-leukocyte interactions, fibrin and tissue factor recruitment into platelet aggregates and thrombus formation (18). Its soluble form, sP-selectin, is released upon platelet and/or endothelial cell activation and measurement of sP-selectin has been proposed as a reliable marker of *in vivo* platelet activation (19).

The aim of our study was to assess, in hospitalized COVID-19 patients, the ability of sP-selectin to predict requirement for mechanical ventilation and death. Next, using whole-blood transcriptional data, we uncovered a platelet activation transcriptional signature associated with critical illness.

Results

Patient characteristics

Two independent cohorts were analyzed for this study. Cohort 1 consisted of 60 COVID-19 patients that were admitted to European Georges Pompidou Hospital and included upon admission. Clinical and biological characteristics are previously reported in (20) and described in **Table 1**. Briefly, median age was 58.5 years (IQR, 49 to 72) and 76.7% were male. Patients were analyzed after a median duration of 6 days (IQR, 4 to 7) after onset of first symptoms. Fever was present in 97% of the patients, and other most common symptoms were cough (83.3%), dyspnea (71.7%), fatigue (70.0%), myalgia (40.0%) and diarrhea (26.7%). Degree of COVID-19 severity was categorized as mild-to-moderate in 35 patients (clinical symptoms associated with dyspnea and requiring a maximum of 3L/min), severe in 10 patients (respiratory distress requiring more than 3 L/min of oxygen and no other organ failure) and critical in 15 patients (respiratory failure requiring mechanical ventilation).

Eleven out of 35 patients with mild-to-moderate disease and 3/10 patients with severe disease experienced clinical worsening and required mechanical ventilation. Thrombotic events were noted in 8 patients (3 mild-to-moderate, 2 severe and 3 critical).

Cohort 2 consisted of 50 patients with various degree of COVID-19 admitted to Cochin hospital and 18 healthy controls, and was described in (21). For the purpose of the present study, only patients with whole-blood transcriptional analysis were included, i.e. 32 COVID-19 patients and 13 healthy controls.

Characteristics of the patients are described in **Table 2**. Median age was 56 years (IQR, 51 to 65) and 78% were male, while median age of healthy controls was 59 years (IQR, 41 to 60) and 77% were male. Patients were analyzed after a median duration of 10 days (IQR, 9 to 11) after onset of first symptoms. Fever was present in 100%, and other most common symptoms were dyspnea (100%), fatigue (32%), cough (97%), myalgia (97%) and diarrhea (34%). Degree of COVID-19 severity was categorized as mild-to-moderate in 11 patients (median oxygen requirement 1.5 L/min), severe in 10 patients (median oxygen requirement 5 L/min) and critical in 11 patients. No patients with mild-to-moderate disease required admission to an ICU, while 5 out of 10 patients with severe disease were eventually admitted to the ICU. Thrombotic events were noted in 3 patients (1 severe and 2 critical).

Increased plasma sP-selectin on admission to the hospital is associated with disease severity and is a predictor of mechanical ventilation and death

Because a high proportion of critically ill patients present clinical and biological evidence of activated coagulation, we measured plasma sP-selectin in Cohort 1. Increased plasma sP-selectin levels on admission to the hospital was significantly associated with critical disease (**Figure 1a**). Moreover, sP-selectin levels were positively correlated with inflammatory parameters, i.e. CRP levels ($r = 0.30$, $p = 0.017$, **Figure 1b**), but not with platelet count ($r = 0.1$, $p = 0.43$, **Figure 1c**),

We next evaluated the ability of sP-selectin, normalized to platelet counts, to predict intubation (**Figure 1d**) or death (**Figure 1e**) using a receiver operating characteristic (ROC) curve. We found that outcomes were significantly associated with higher sP-selectin values and significant area under the curve (AUC = 0.67, $p = 0.028$ for intubation and AUC = 0.74, $p = 0.0047$ for death). An optimal cutoff value of 150 NC (normalized concentration: concentration in pg/mL normalized to 10^6 platelets/mL) was predictive of intubation with 66% sensitivity, 61% specificity, 66% negative predictive value (NPV) and 61% positive predictive value (PPV), and of death with 82% sensitivity, 60% specificity, 90% NPV and 55% PPV. Together, these data suggested that sP-selectin, a reliable marker of platelet activation, was strongly associated with death and may identify patients at higher risk of admission to the ICU.

Critically ill COVID-19 patients display a dysregulated primary hemostasis transcriptional signature

We previously performed a whole-blood immunological transcriptional characterization on 32 patients with laboratory-confirmed COVID-19 displaying various disease severity (cohort 2) (21). We therefore next analyzed genes involved in hemostasis using an unbiased gene set enrichment analysis comparing severe to critical COVID-19 patients. We found that critically ill patients displayed an elevated expression of genes related to primary hemostasis (**Figure 2a**). Hierarchical clustering identified genes that were upregulated in a grade-dependent manner, thereby driving the hemostasis signature in critically ill patients (**Figure 2b**). These genes included *ITG2AB*, *GP1BB*, *PPBP* and *SELPLG* (**Figure 3a**), which

recapitulated multiple steps of platelet activation (22,23). Importantly, and similarly to sP-selectin, RNA levels more strongly correlated with CRP level (**Figure 3b**) than with platelet counts (**Figure 3c**).

We next evaluated the ability of these genes to predict clinical outcome after normalization with platelets. Among patients that did not initially require critical care upon inclusion (n = 21), 5 (24%) presented respiratory failure afterwards requiring mechanical ventilation (**Table 2**). *PPBP* and *SELPLG* were found to be the best predictor of clinical worsening, with an AUC of 0.8 for both outcomes ($p = 0.048$) (**Figure 4a, b and c**). An optimal cutoff value for *PPBP* of 5 NRC (normalized RNA counts : counts normalized to 10^6 platelets/mL) was predictive of intubation with 100% sensitivity, 63% specificity, 100% NPV and 45% PPV, while a cutoff value of 2.65 NRC for *SELPLG* was predictive of intubation with 100% sensitivity, 69% specificity, 100% NPV and 50% PPV. Overall, these markers provide excellent negative predictive values, with no patient below the threshold value requiring future mechanical ventilation.

Discussion

Dysregulated hemostasis is emerging as a key factor in COVID-19 pathogenesis and severity. The present study provides new insights into the contribution of platelets to disease severity with the identification of a unique hemostasis signature in critically ill patients. We identified sP-selectin as a soluble marker associated with death, and elevated *SELPLG* and *PPBP* RNA levels as strong negative predictors of mechanical ventilation in hospitalized patients.

Recently, altered platelet gene expression and elevated sP-selectin were reported in COVID-19 patients (24,25). Our study supports these findings and highlights the key role of primary hemostasis gene-expression signature in critical patients, especially the specific platelet chemokine-encoding gene *PPBP* and the gene encoding for the P-selectin glycoprotein ligand-1 (PSGL-1), *SELPLG*. *PPBP* and *SELPLG* gene expression could also predict clinical outcome. Pro-platelet basic protein (PPBP), also known as CXCL7, is the most abundant platelet chemokine and (22,26) expressed within platelets as an inactive precursor and activated after cleavage during thrombus formation by enzymes released by neutrophils, while PSGL-1 is expressed by leukocytes, allowing the formation of platelet-leukocyte conjugates and adhesion of leukocytes to activated endothelium through its interaction with P- or E-selectin (22,27). PPBP has been shown to be essential for neutrophil migration into the thrombus (28) and the formation of platelet-neutrophils aggregates (18), since its receptor, CXC chemokine receptor 2 (CXCR2), is expressed on the surface of neutrophil. Supporting thus, we previously observed increased *CXCR2* expression in severe COVID-19 patients (21). Murine models of acute lung injury showed that deletion of *PPBP* protected the mice from lung disease (26), as well as blocking platelet activation (29,30). Overall, our data support a model whereby platelet activation induces PPBP release followed by neutrophil attraction into sites of injury, precipitating organ injury and failure. This hypothesis is also supported by increased sP-selectin in most severe patients, since P-selectin on the surface of platelets promotes the formation of platelet-neutrophil or monocyte aggregates (27), via binding to its ligand PSGL-1 (encoded by *SELPLG*) expressed on the surface of leukocytes (31). Accordingly, increase in platelet-monocyte aggregates has been

recently described in severe COVID-19 (32), a mechanism that was effectively blocked by platelet P-selectin neutralization.

The identified hemostasis gene-signature also included *ITG2AB* and *GP1BB*. *ITG2AB* encodes integrin alpha-IIb subunit, a member of the receptor complex integrin alpha-IIb/beta-3 ($\alpha\text{IIb}\beta\text{3}$), which is the receptor for fibrinogen, plasminogen, prothrombin and thrombospondin (23). Importantly, binding of integrin $\alpha\text{IIb}\beta\text{3}$ to fibrinogen forms bonds between adjacent platelets, an essential step for clot formation. Finally, *GP1BB* encodes glycoprotein Ib, the receptor for vWF, and mediates platelet adhesion in the arterial circulation (23,33). Altogether, these upregulated genes recapitulate multiple steps of platelet activation (22,23).

In addition to their role in primary hemostasis, platelets are an integral part of the immune response to pathogens. They possess a wide array of microbial sensors and secrete inflammatory mediators upon activation, hence amplifying innate immune responses. The functional interdependence and the coordinated activation of both processes, designated as thrombo-inflammation, may drive adverse effects such as thrombosis, multiple organ failure and death (34). We and others have shown specific inflammatory signatures that characterized severe and critical from mild-to-moderate COVID-19 patients (e.g. type I interferon pathways, NF- κ B pathways). Intriguingly, we found that the hemostasis gene signature was able to further distinguish critical from severe COVID-19 patients, underlying the hypothesis that deregulated hemostasis, in a continuum with inflammation, could drive COVID-19 progression into critical disease. Viral infection and sepsis models have been associated with platelet gene expression and functional alterations (35,36). However, some unique features may characterize SARS-CoV-2-mediated platelet dysregulation. Manne *et al.* demonstrated that platelet gene-expression profile was unique in COVID-19 patients in comparison to H1N1 pandemic patients (25). Moreover, thrombocytopenia, another feature of sepsis during viral infection (37,38), was not observed in COVID-19 patients. Conversely, we and others found that platelets counts tended to increase with disease severity, supporting the unique aspects of platelet dysregulation in COVID-19 critical patients.

Identification of such biomarkers of clinical outcome can have strong impact on clinical practice and help provide novel therapeutic rationale. Prediction of illness trajectories after the onset of symptoms is difficult but remains critical to more accurately identify patients that will require ICU. Our findings also suggest that a subset of patient may benefit from drugs preventing platelet activation such as antiplatelet agents (e.g. NCT04365309 ; NCT04363840) or P-selectin inhibitors such as crizanlizumab (e.g. NCT04435184) (39). This strategy, in combination with anti-inflammatory drugs (glucocorticoids, anti-interleukin-6) could have substantial impact on thrombo-inflammation, which seems to drive COVID-19 severity.

Our study has some limitations. We used two independent cohorts with a small sample size performed each in a single center. The two cohorts were not homogenous in regards to timing of measurements and severity of patients. Also, the study was not designed as a longitudinal study, so no sequential measurement was available. Serial measurement of identified biomarkers over time will be important to

more precisely define the optimal time window for their measurement, and hence improve their prediction performance. Moreover, our transcriptomic analysis was performed on whole-blood RNA, so we cannot evaluate the contribution of each cell population to hemostasis dysregulation. Separate transcriptional profiling of platelets versus other circulating populations may provide further insights into the contribution of each population to thrombo-inflammation and dissect the mechanisms of platelet–neutrophil or platelet–monocyte aggregates.

This exploratory study sheds light on dysregulated hemostasis and the role of thrombo-inflammation in critical patients. We identified platelet activation marker sP-selectin, *SELPLG* and *PPBP* as potential biomarkers of critical worsening and provide rationale for evaluating sP-selectin blockade or anti-platelet drugs in most severe COVID-19 patients. Additional studies designed to test the performance of these biomarkers are required to both validate our findings and optimize their ability to predict progression to critical disease.

Methods

Cohorts

Two independent cohorts were analyzed for this study. Data used for the analysis of soluble P-selectin's ability to predict admission to ICU were extracted from a non-interventional study that was conducted at European Georges Pompidou Hospital (Paris, France) and partially described in (20) (Cohort 1). Briefly, Cohort 1 included consecutive patients with SARS-CoV-2 infection. Inclusion criteria were patients over 18 years of age, with a proven SARS-CoV-2 infection, which presented to the emergency department with hospitalization criteria. Patients were then hospitalized into conventional wards or directly to the ICU. For all patients, baseline characteristics (demographic, treatment, clinical, cardiovascular risk factors and body mass index) and biological data were retrieved from the medical records using a standardized data collection.

Cohort 2 (21) was conducted between March 19, 2020 and April 3, 2020 in Cochin Hospital (Paris, France), in the setting of the local RADIPEM biological samples collection derived from samples collected in routine care. Inclusion criteria for COVID-19 inpatients were: age between 18 and 80 years old, diagnosis of COVID-19 according to WHO interim guidance, and positive SARS-CoV-2 RT-PCR testing on a respiratory sample (nasopharyngeal swab or invasive respiratory sample). Detailed clinical and immunological characterization of the cohort was previously described in (21). Epidemiological, demographic, clinical, laboratory, treatment, and outcome data were extracted from electronic medical records using a standardized data collection form.

The severity of COVID-19 was classified at the time of inclusion based on the adaptation of the Sixth Revised Trial Version of the Novel Coronavirus Pneumonia Diagnosis and Treatment Guidance and described in (21) .

Soluble P-selectin measurement

Soluble P-selectin quantification was performed on Cohort 1. Plasmas were collected on 0.129 M trisodium citrate tubes (9NC BD Vacutainer, Plymouth, UK). Plasma poor platelet was obtained after centrifugation twice at 2500 g for 15 minutes. PPP was frozen after a second centrifugation at 2500 g for 15 minutes and stored at -80°C until analysis of vascular markers. Soluble P-selectin were quantified in PPP with a Human Magnetic Luminex Assay from R&D systems (Lille, France). Data were assessed with the Bio-Plex 200 using the Bio-Plex Manager 5.0 software (Bio-Rad, Marnes-la-Coquette, France). Normalized concentration (NC) used to calculate ROC AUC p -values represents sP-selectin concentration in pg/ml normalized to platelet numbers (10^6 /mL).

Gene expression analysis

Detailed methods was previously reported in Hadjadj et al. (21). Briefly, we analyzed 100 ng (5 μ l) of total RNA from each sample using the Nanostring Human Immunology kit v2 according to manufacturer's instructions. Raw RNA counts were adjusted using five housekeeping genes selected from the 15 candidate control genes provided by Nanostring, following the geNorm method. For gene set enrichment analysis (GSEA), genes were ordered by t-statistic from unpaired t-test comparing normalized RNA levels of severe vs critical patients, and then fed to the gene set enrichment algorithm (GSEA version 4.0.3, Broad Institute), along with a pathway data set built from the Nanostring Immunology panel version 2 annotation file. Parameters were set as follows: method, pre ranked gene list; number of permutations, 2,000; enrichment statistic, classic; min set size, 5; max set size, 200; and all other parameters as default. Hierarchical clustering of genes belonging to the hemostasis gene set was performed using hclust with default distance matrix. Heatmap displaying genes that are upregulated in a grade-dependent manner was obtained using pheatmap (package pheatmap), with data centered to 0 and scaled to unit variance for each gene. Normalized RNA count (NRC) used to calculate ROC AUC p -values represents adjusted RNA count (to house keeping genes) normalized to platelet numbers (10^9 /L).

Statistical analyses

Quantitative variables were compared among groups using Kurskal–Wallis test followed by Dunn's post test, while quantitative variables were compared using the χ^2 test of independence. Correlations coefficients and p -values were assessed using Spearman's method. ROC AUC p -values were determined using Hanley's method. Optimal thresholds were determined by maximization of Youden's index. All analyses were two-sided and a p -value smaller than 0.05 was considered statistically significant. Statistical analyses were performed using R v. 3.4.3 (CRAN).

Declarations

Ethics approval

Both studies conformed to the principles outlined in the Declaration of Helsinki, and received approval by the appropriate Institutional Review Board (CPP2020-04-048/2020-A01048-31/ 20.04.21.49318).

Availability of data and material

All data are available upon reasonable request.

Competing interests

None of the authors has a relevant competing interest

Authors' contributions

NY, JB, JH, DMS and BT conceived and designed the study and had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. NY, JB, JH, LB, BC, DMS and BT performed research. NY, JB, DMS and BT drafted the paper. NY, JB, NG, RC, DD, DMS and BT did the analysis and all authors critically revised the manuscript for important intellectual content and gave final approval for the version to be published. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Tables

The tables can be accessed in the Supplementary Files.

Figures

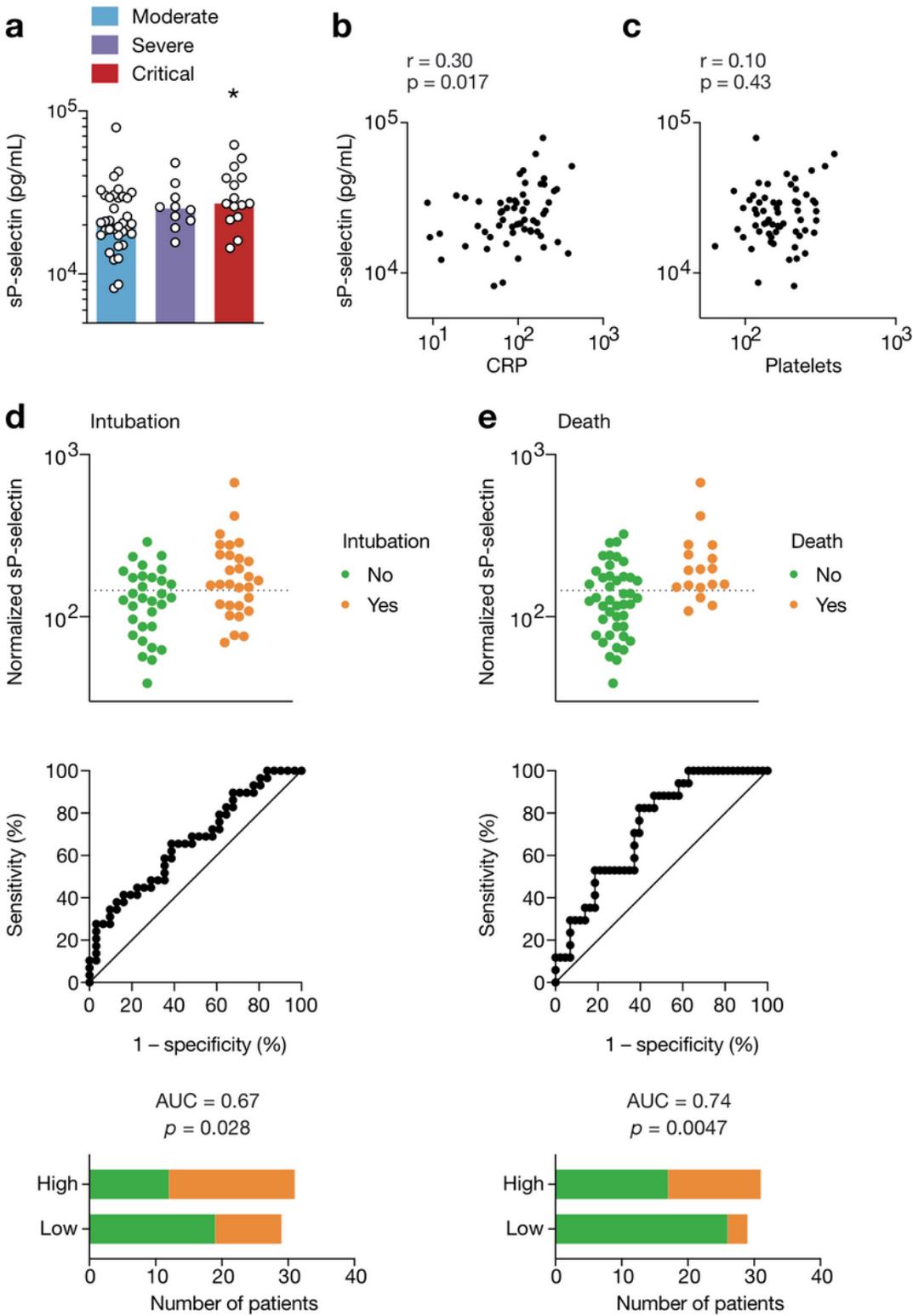


Figure 1

sP-selectin as a marker of COVID-19 severity and a predictor of intubation and death. a, Soluble P-selectin was measured in plasma from patients from Cohort 1 and plotted according to severity grade. Groups: mild-to-moderate (n = 35), severe (n = 10) and critical (n = 15). *p < 0.05 (Kruskal–Wallis test followed by Dunn’s post-test). b,c, sP-selectin correlation with CRP levels (b) or platelet counts (c). r and p-values were determined using Spearman’s correlation test. d, e, sP-selectin normalized to platelet counts as a predictor of intubation (d) or death (e). ROC curves with area under the curve (AUC) and associated p-values are shown. Optimal threshold was determined by maximization of Youden’s index, and represented by a dashed line. Bar graphs indicate the number of patients in each group depending on normalized sP-selectin levels with respect to the optimal threshold. Groups: no intubation (n = 31), intubation (n = 29), no death (n = 43), death (n = 17).

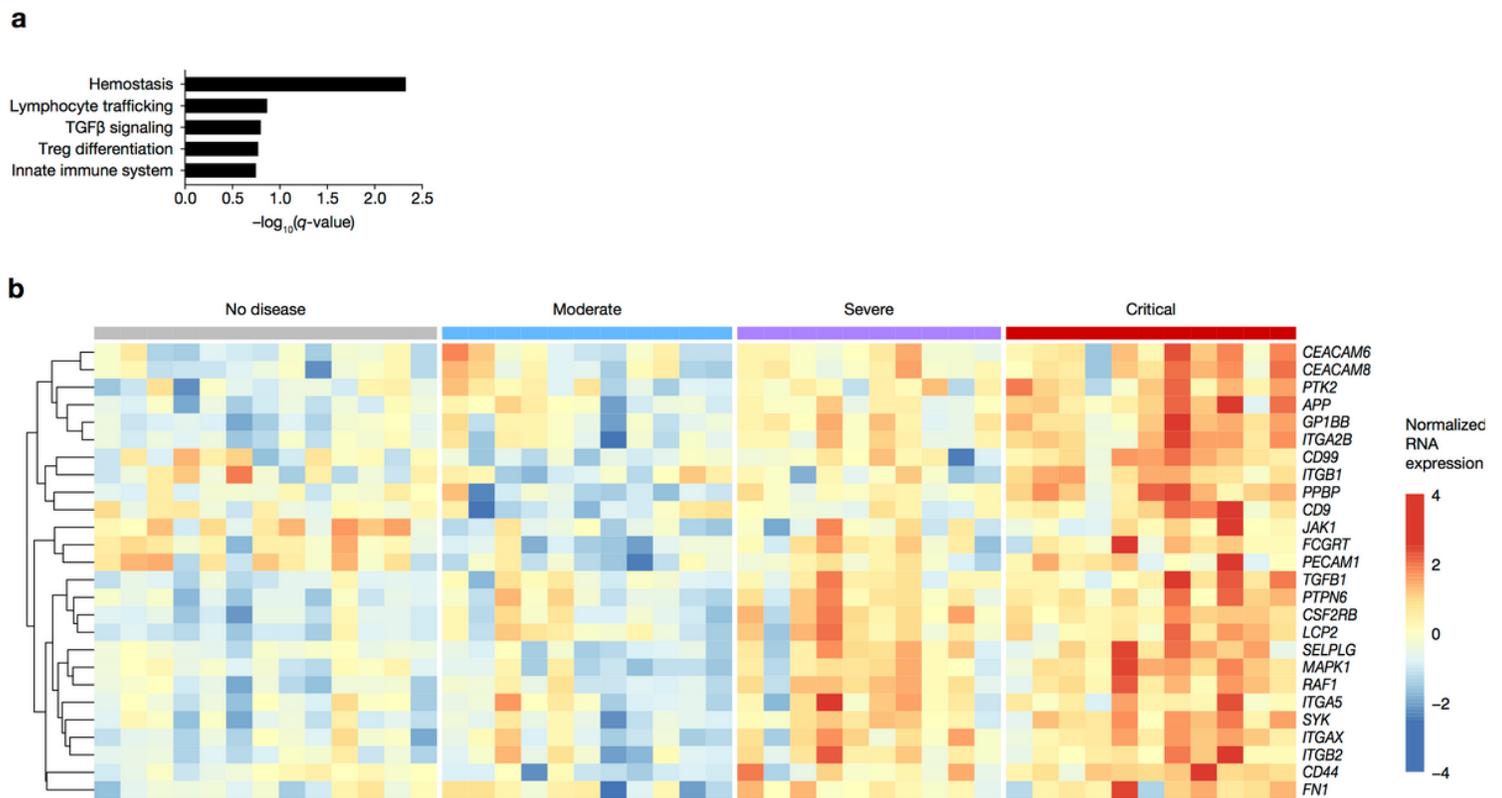


Figure 2

Unbiased transcriptomic analysis reveals hemostasis-related genes as upregulated in critically ill COVID-19 patients. RNA from patients of Cohort 2 was extracted from whole blood and RNA counts of 594 genes were determined by direct probe hybridization using the Nanostring nCounter Human Immunology v2 kit. a, Gene-set enrichment analysis was performed after ranking genes according to their differential expression in severe vs critical patients. Shown are pathways significantly enriched (false discovery rate < 0.2). b, Heatmap representation of genes of the hemostasis pathway that are upregulated in a severity grade-dependent manner (determined by hierarchical clustering). Up-regulated genes are shown in red and down-regulated genes in blue. Groups: healthy controls (n = 13), mild-to-moderate (n = 11), severe (n = 10) and critical (n = 11).

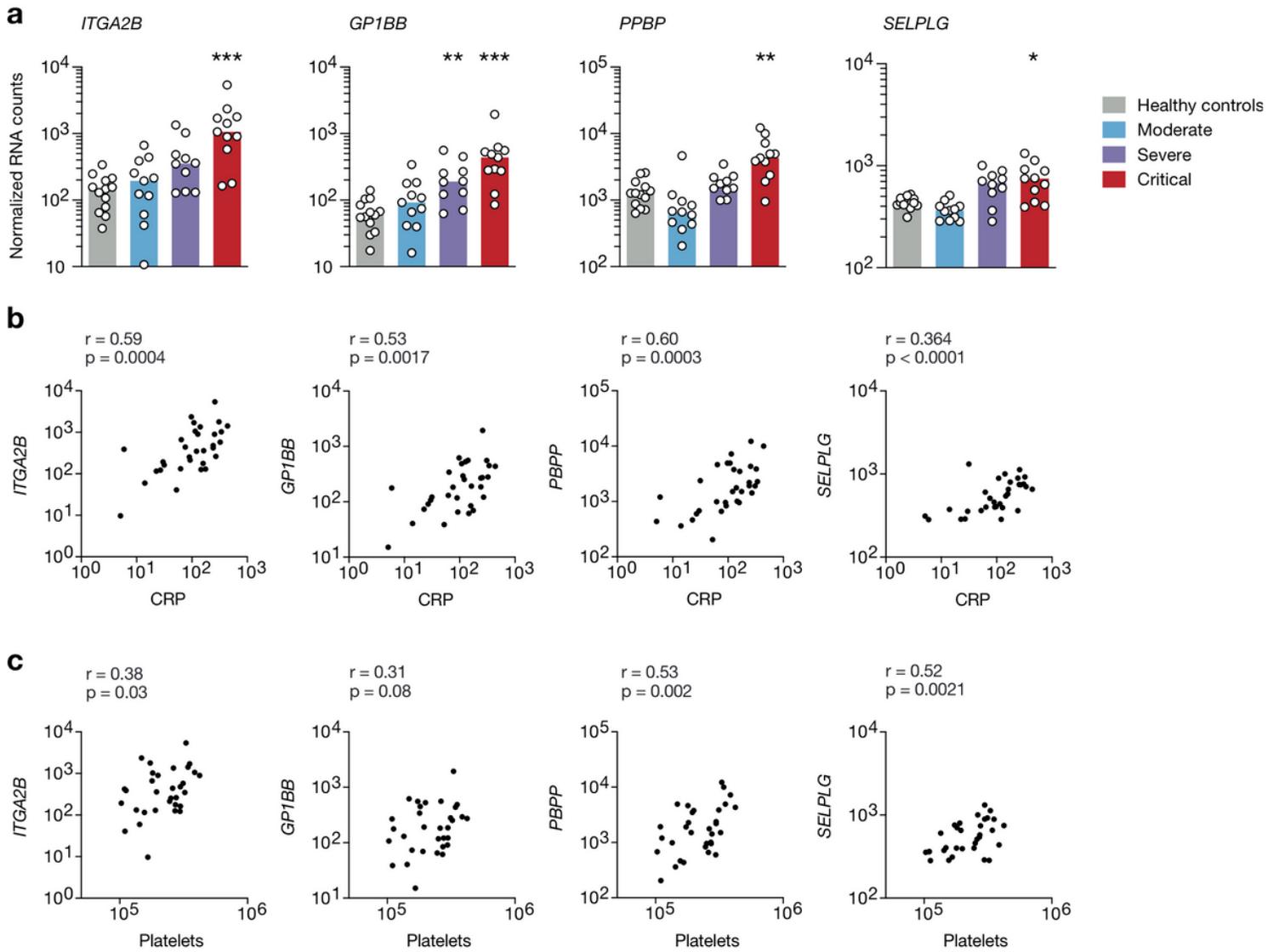


Figure 3

Platelet gene dysregulation in critically ill patients. a, Normalized RNA expression of ITGA2B, GP1BB, PPBP, SELPLG depending on severity grade. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (Kruskal–Wallis test followed by Dunn’s post-test). b,c, Gene expression correlation with CRP levels (b) or platelet counts (c). r and p -values were determined using Spearman’s correlation test. Groups: healthy controls ($n = 13$), mild-to-moderate ($n = 11$), severe ($n = 10$) and critical ($n = 11$).

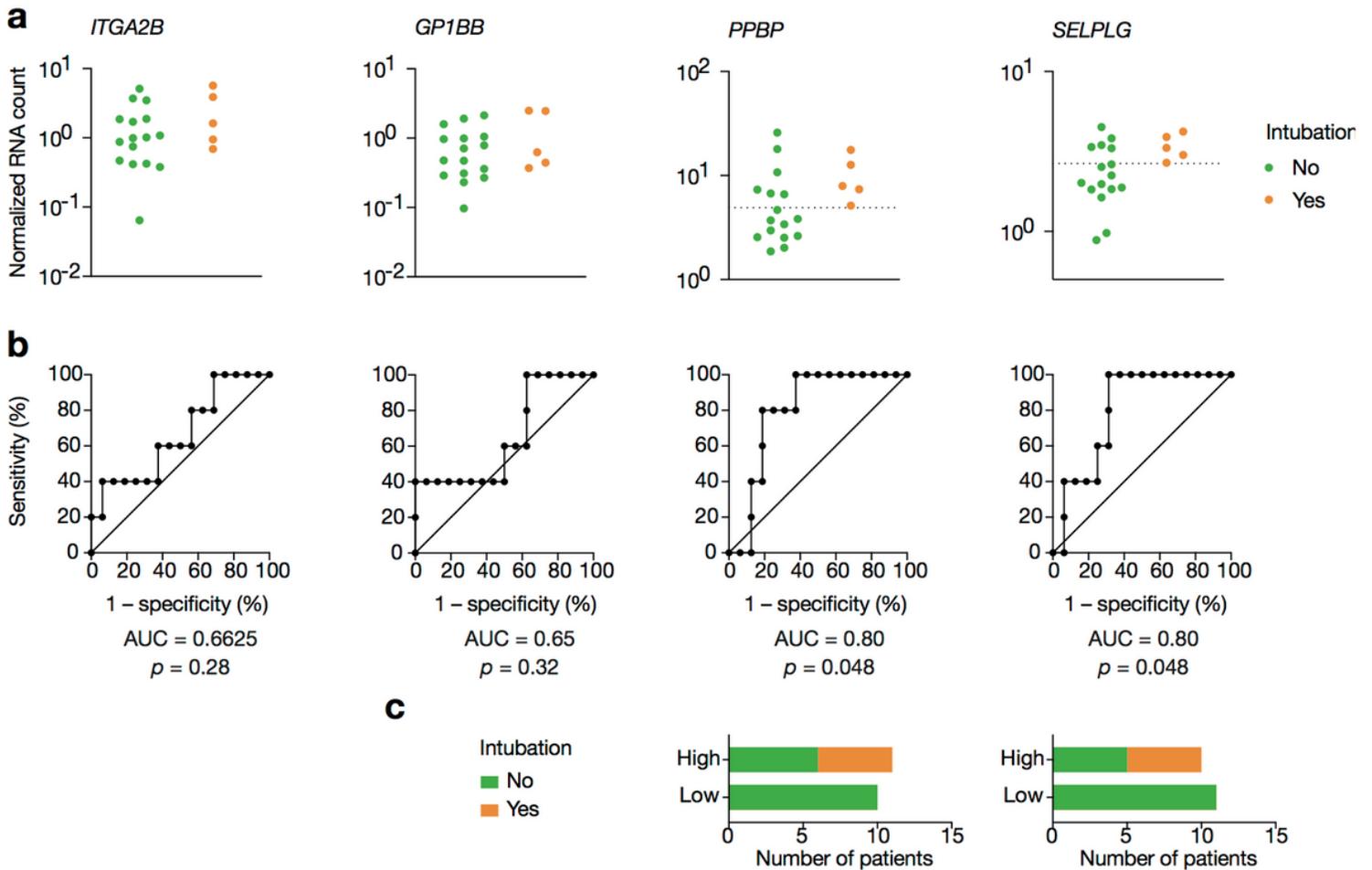


Figure 4

Prediction potential of hemostasis genes for intubation. a, RNA levels of non-severe patients at admission depending on their future aggravation, normalized to platelet counts. Dashed lines represent optimal threshold obtained by maximization of Youden's index for significant AUCs. b, Associated ROC curves, with area under the curve (AUC) and associated p-values. c, Bar graphs indicating the number of patients in each group depending on normalized RNA levels of a single gene with respect to the optimal threshold. Groups: no intubation (n = 16) and intubation (n = 5).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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